A RAPID PROCEDURE FOR THE DETECTION OF AFLATOXINS IN FIELD-COLLECTED COTTONSEEDS BY THIN-LAYER CHROMATOGRAPHY

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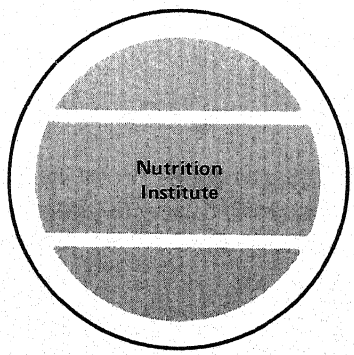

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A RAPID PROCEDURE FOR THE DETECTION OF AFLATOXINS IN FIELD-COLLECTED COTTONSEEDS BY THIN-LAYER CHROMATOGRAPHY

By George V. Merola, Renato J. Ferretti, Paul B. Marsh, and Marion E. Simpson [1/]

SUMMARY

A unique miniplate procedure is described for the detection of aflatoxins in cottonseeds. It is intended for use in field studies on the control of aflatoxin contamination in cottonseeds before harvest. The original extract of the seeds is chromatographed directly without undergoing any preliminary purification. A crushed seed-meat sample is shaken with three times its weight of chloroform:acetonitrile (1:2) and one-half its weight of 10 percent aqueous ferric chloride ($FeCl_3$), after which the extract is chromatographed on a 2-by 3-inch thin-layer chromatography (TLC) plate with a 3/4-inch band of aluminum oxide (Al_2O_3) at the bottom and a 2 1/4-inch band of silica gel on the remainder of the plate. The developing solvent is diethyl ether: methanol:water (96:3:1).

Seeds from bolls that were incubated in pure culture with other common boll-rot fungi beside Aspergillus flavus exhibited no interfering fluorescent spots on the miniplates that could be confused with the aflatoxins. A series of seeds segregated from field-collected seed cotton in such a manner that each seed was known to be infected with A. flavus exhibited extreme variability in aflatoxin content from one seed to another.

GENERAL BACKGROUND

Previous research has demonstrated that cottonseeds from most U.S. cotton-growing locations are virtually if not entirely free of aflatoxins at harvest (6, 7, 13).[2/] In a few locations, however, contamination with aflatoxins B_1 and B_2 has occurred in association with a boll rot caused by the aflatoxin-producing fungus Aspergillus flavus (6, 7, 9). Evidence of this boll rot is easily detectable in ginned lint, seed cotton[3/], or fuzzy seeds

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1/ Chemists, plant physiologist, and plant pathologist, respectively, Nutrition Institute, Northeastern Region, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md. 20705

2/ Italic numbers in parentheses refer to Literature Cited, p. 9.

3/ "Seed cotton" designates the seed as it occurs on the plant with fiber attached, whereas "cottonseed" or "fuzzy seed" refers to the seed after removal of the fiber by ginning.

by the presence of a characteristic bright greenish-yellow (BGY) fluorescence in the lint or fuzz (9). The fluorescing pigment is formed before boll opening or simultaneously with opening, and is produced from kojic acid, a metabolite of A. flavus, in the presence of peroxidase, a constituent of the fiber (8). Research is currently underway to find means to control or to alleviate the field problem of aflatoxins in cotton, and the miniplate method described here is intended to aid that research.

In field research on the cottonseed-aflatoxin problem, large numbers of samples must be tested. Individual samples sometimes comprise the seeds from only a few bolls. In the test, simplicity is much more important than an extremely low limit of detection. In part, the need is met by the use of the BGY fluorescence in the seed fuzz and fiber as a detection device. This fluorescence is reliably diagnostic for A. flavus boll rot (10) and frequently is associated with aflatoxins in the seeds (6, 7, 13). However, aflatoxin contamination can occur in a particular sample in the absence of the fluorescence or fail to occur in its presence (7).

Data recently presented (13) and relevant to the point mentioned just above are summarized here to show the relation between BGY fluorescence and aflatoxin in 161 commercial seed cotton samples from the crop of 1971. The three samples with aflatoxins in the seeds from the non-BGY-fluorescing locks also had aflatoxins in the seeds from the BGY-fluorescing locks. Aqueous-extract fiber pH of all samples was in the range 5.8 - 7.6, indicating little or no exposure to damp conditions after harvest.

161 seed cotton samples collected		
65 samples had no BGY-fluorescing locks	96 samples had some BGY-fluorescing locks	
No samples had detectable aflatoxins in the seeds.	In BGY-fluorescing locks, 51 samples had detectable aflatoxins in their seeds and 45 samples did not.	In non-BGY fluorescing locks, 3 samples had detectable aflatoxins in their seeds and 93 samples did not.

A simple, semiquantitative confirmatory procedure is needed to provide certain proof of aflatoxins in the seeds. The present paper describes such a method.

The possibility that infection with other boll-rot fungi beside A. flavus might interfere with aflatoxin determinations had to be considered. This was particularly true for Aspergillus niger because this fungus is a very common cause of boll rot and field infection of cottonseeds in the Far West (13) and because a previously published report states that some black Aspergilli can produce a fluorescing material that chromatographs in some systems like aflatoxin B_1 (4), even though its identity as an aflatoxin seems not to have been clearly established.

Methods of analysis for aflatoxin in various commodities have been broadly reviewed by Goldblatt (3), Stoloff,[4/] and Detroy and others (2). In most methods for estimating aflatoxins in cottonseeds, the seeds are first dehulled; then the meats are ground, extracted with solvent, and the solution partially purified of nonaflatoxin components. The extract, thus prepared, is chromatographed on a thin-layer plate and compared with aflatoxin standards. Abbreviated chromatographic methods involving minicolumns and providing semi-quantitative estimates of aflatoxin levels have been described by Cucullu and others (1), Pons and others (11, 12), and Velasco (14, 15). These methods are intermediate in time requirements and have obvious usefulness.

Lee (5) found that the swelling that took place when water was added to a peanut sample caused disruption of the cell structure and that rapid solution of the aflatoxin in chloroform would then occur. Considerable amounts of oil and pigments were also extracted. Cucullu and others (1) used acetonitrile:water (80:20) to extract aflatoxin rapidly from cottonseeds with very little extraction of oil, while Yatsu and others (16) used ferric ions to remove gossypol pigments from cottonseed miscella.

APPROACH TO THE PROBLEM

Starting with the above facts, extractions were made with various combinations of acetonitrile, chloroform, and aqueous ferric chloride in an effort to find a combination that would provide rapid extraction of aflatoxins with minimum extraction of oil. A large microscope slide, partly coated with silica gel and partly with aluminum oxide, was used as a TLC miniplate (fig. 1).

Various types of Al_2O_3 were tested to determine the amount of streaking produced and their ability to adsorb and retain impurities other than aflatoxins.

The combination of extract solvent, aqueous $FeCl_3$, Al_2O_3, silica gel, and development solvent that was finally adopted eliminated the need for a preliminary cleanup procedure. The fluorescent and nonfluorescent substances in the extract, present especially in highly infected seeds, did not interfere, because they either remained at the origin or traveled with or near the solvent front. By using aqueous $FeCl_3$ instead of water, a clearer sample extract was obtained, and the complexed pigments remained on the Al_2O_3 layer. In some instances, this sample extract could be spotted directly on large S & S silica gel plates without interference from the complexed pigments. When a sample of pure gossypol, complexed or not, was spotted on the Al_2O_3 layer of the miniplate and developed, the gossypol remained at the origin.

A standard solution containing 5, 1.5, 5, and 1.5 µg per ml of aflatoxin B_1, B_2, G_1, and G_2, respectively, when chromatographed, produced four discrete spots on the miniplate or on the larger plates. Only aflatoxin B_1 was estimated for the present tests since the G aflatoxins were absent and B_2 was present only in lower amounts than B_1.

4/ Stoloff, L. Analytical methods for mycotoxins. Presented at the ACVT annual scientific session, July 21, 1971, 39 pp. (mimeo.)

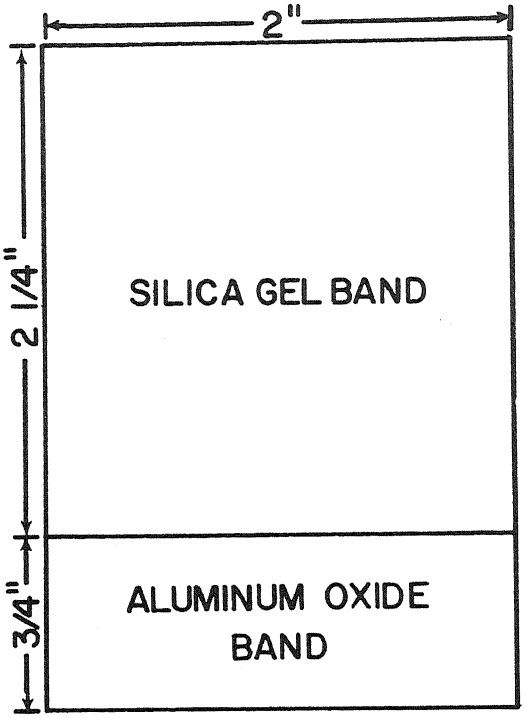

Figure 1.--TLC miniplate prepared for use in analysis of cottonseeds for aflatoxins

APPARATUS

1. Mechanical shaker.
2. Long-wave ultraviolet lamp. (Blak-Ray Model B-100A, Ultra Violet Products, San Gabriel, Calif.)
3. Microliter syringe: 50 µl. (Hamilton.)
4. Developing tank. One-half pint, wide-mouth Ball jar with glass plate cover or equivalent-size Berzelius beaker.
5. Miniplates: 2-by 3-in (50-by 75-mm) microscope slides. (Fisher Scientific, Pittsburgh, Pa.)
6. Adsorbent applicator. Rigid polyethylene tubing, 7-mm O.D. by 5-mm I.D., 12 cm in length with a 2-ml rubber bulb attached and with the tip cut at an angle of 45°.
7. Hair dryer. (Model 202, John Oster Co., Milwaukee, Wis.)
8. Polyethylene tubing. 0.038-in O.D. by 0.023-in I.D. (Catalog No. 14-170-12B, Fisher Scientific, Pittsburgh, Pa.)
9. U.S. Standard Sieve Series. No. 10, Tyler equivalent 9 mesh.
10. Silica gel ready plates: acid-fast, G-1500, 20 by 20 cm. (Schleicher and Schuell, Keene, N.H.)

REAGENTS

1. Solvents: reagent-grade acetonitrile, benzene, chloroform, ether, and methanol.
2. Extraction solvent: chloroform:acetonitrile (1:2).
3. Development solvent: diethyl ether:methanol:water (96:3:1).
4. Silica gel (MN silica gel G). (Brinkmann Instruments, Westbury, N.Y.)
5. Aluminum oxide, neutral (type T) 1101. (Brinkmann Instruments, Westbury, N. Y.)
6. Ferric chloride: reagent grade.
7. Standard aflatoxin: 0.5 µg B_1 per ml.

PREPARATION OF MINIPLATES

The two adsorbent layers are applied to six 2-by 3-in microscope slides in the following manner: 6 g of MN silica gel G and 12 ml of distilled water are mixed thoroughly in a small beaker, and the resulting slurry is drawn into the plastic applicator by an attached rubber bulb. Next, the slurry is spread in a straight line starting three-fourths of an inch from the bottom and covering approximately 2 1/4 in of the surface of each of the six slides.

After several minutes, a slurry of 3 g Al_2O_3 (type T) in 4.5 ml of distilled water is applied in the same manner to the remaining three-fourths of an inch at the bottom of each slide, making sure that the two adsorbents touch each other. Shaking the miniplate gently after each adsorbent has been applied distributes the slurry evenly over the surface. The miniplates thus prepared are allowed to dry overnight at room temperature (fig. 1). Other methods of application of the adsorbents to the miniplates may be satisfactory. The thickness of the adsorbent layers is not critical. Activation of the plates at an elevated temperature is not required before use.

PROCEDURE

A weighed sample of approximately 7 g of fuzzy cottonseed is placed into a small plastic bag and then crushed with a hammer. A no. 10 sieve is used to separate the crushed meats from the hulls and lint. To the crushed-meat sample is added three times its weight of extraction solvent, chloroform: acetonitrile (1:2), and one-half its weight of 10 percent aqueous ferric chloride. The sample is then shaken for one-half hour and allowed to settle for several minutes. No filtering is necessary.

A 6-in length of polyethylene tubing (0.023-in I.D.) is attached to a 50 µl Hamilton syringe, and 30 µl of sample extract is drawn into the tubing. Under a hair dryer, the 30 µl of extract is spotted 2-4 µl at a time on the lower, Al_2O_3 layer of the miniplate, while the spot is kept approximately 5 mm or less in diameter. Four samples and one standard containing 1 ng of aflatoxin B_1 are spotted on each plate. The plate is carefully placed into the half-pint Ball jar and developed for 8 min in diethyl ether:methanol:water (96:3:1) (14). Then the plate is removed, dried under a hair dryer, and examined under long-wave ultraviolet light. If aflatoxin B_1 is present, the blue fluorescent spot has moved approximately 1 in into the silica gel layer

on the miniplate. Other aflatoxins, if present, have a lower R_f. Oils and pigments in the sample extract either remain at the point of origin or travel with or near the solvent front as the solvent moves up the plate.

When a more quantitative estimate of the aflatoxin level is desired, a visual estimate is made from the miniplate, and an aliquot of the extract is diluted accordingly with benzene:acetonitrile (98:2). This diluted extract is then spotted on a 20- by 20-cm S & S silica gel plate and developed in an unlined tank with the same solvent system used for the miniplates. Activation of these plates by heating is not required, nor is any further purification of the samples necessary.

NONINTERFERENCE FROM INFECTIONS WITH FUNGI OTHER THAN ASPERGILLUS FLAVUS

To check on possible interferences with the miniplate procedure that might be caused by metabolites produced by fungi other than A. flavus, cotton bolls were incubated in live condition for 1 week with certain fungi that are known to infect cottonseed before harvest. These seeds were then tested for aflatoxins. The fungi used were A. niger, Fusarium, Rhizopus stolonifer, Alternaria, and Diplodia gossypina.

In all cases except the Diplodia, no fluorescing spots appeared at any position on the miniplate. With Diplodia, a blue-fluorescing spot appeared near the solvent front in a position considerably above the aflatoxin B_1. This spot could not have been confused with it nor with aflatoxin B_2, the only two aflatoxins that have been reported in cottonseed at harvest. When the Diplodia and A. flavus extracts were combined and chromatographed on the miniplate, the two fluorescent spots were clearly separated.

Of particular interest was the fact that the miniplate chromatograms of the seeds from bolls incubated with A. niger did not show any fluorescing spots, since this fungus is an extremely common cause of infection in cottonseeds in areas where A. flavus is also found (13).

VARIABILITY IN AFLATOXIN LEVELS AMONG SEEDS KNOWN TO BE INFECTED WITH A. FLAVUS

Extreme variability in aflatoxin levels from one seed to the next has been known for some time to occur in field samples of cottonseeds. A simple and obvious explanation of this phenomenon might be merely that some seeds become infected in the field while others do not. In actuality, however, such variability very probably occurs even within a group of field-collected seeds in which each seed is known to be infected with A. flavus. Evidence for this conclusion has come from analysis of individual seeds from BGY-fluorescing locks taken from nine samples of commercial seed cotton (13).

In the experiment of table 1, seeds from the field-collected BGY-
scing locks were incubated for 4 days on water agar in a standard
- procedure for micro-organisms (6). Then, seeds that showed an

outgrowth of A. flavus were dried and analyzed for aflatoxins. Each seed was carefully and individually dehulled, and the meat was weighed and placed in a vial. An amount of solvent, chloroform:acetonitrile (1:2), equal to five times the weight of the seed meat, was added, the meat crushed with a stirring rod, half its weight of 10 percent aqueous $FeCl_3$ added, and the mixture stirred. After 5 minutes, 10 µl of the extract were spotted on a miniplate and developed as usual. From the intensity of the B_1 fluorescent spot, the sample was diluted accordingly (usually 10 µl to 0.5 ml) and then analyzed on the silica gel plates. The variation in aflatoxin level among individual seeds specifically known to be infected with A. flavus was obviously extreme (table 1). Some readers might think that the nondetectable aflatoxin values in the table could have resulted from destruction of previously produced aflatoxin by secondary organisms also present. Actually, no secondary organisms were visibly present in these seeds.

TABLE 1.--Aflatoxin B_1 levels in individual A. flavus-infected seeds from locks in commercial cotton with BGY fluorescence in fiber (13)

Sample number	No. of seeds	Aflatoxin B_1 level in individual seeds (ppb)[1/]		
55	3	---	2,500,000	---
60	2	---	---	
61	3	150,000	100,000	100,000
122	1	200,000		
143	2	35,000	7,500,000	
150	2	---	150,000	
232	1	2,500		
239	2	2,000,000	25,000	
243	2	5,000,000	---	

1/ Dashes indicate seeds that were analyzed without finding detectable aflatoxins present.

DISCUSSION

The extraction solvent and the development solvent used in this study did not cause the aflatoxin spots on the miniplate to fade after development. By using a ratio of 1 g of sample to 3 ml of extraction solvent and by spotting 30 µl of this extract, the limit of detection on the original extract was 100 ppb, because 1 ng of aflatoxin B_1 is the minimum amount that can be detected on the miniplate. Greater sensitivity can be obtained if an evaporation step is included. On several samples, 1 ml of the original extract was evaporated to dryness under nitrogen in a 70° C water bath; the residue was dissolved in 0.1 ml of benzene:acetonitrile (98:2); and then 10 µl was spotted. This increased the sensitivity to 30 ppb. Preliminary tests showed that preparative amounts of relatively pure aflatoxin B_1 could be obtained when a concentrated cottonseed sample extract was streaked across the Al_2O_3 layer, developed with ether:methanol:water (96:3:1), and the fluorescent aflatoxin B_1 band in the silica gel layers scraped off and eluted with chloroform.

The Al_2O_3 neutral (type T) specified in the miniplate method was far superior to other adsorbents tested in retaining most impurities. These other adsorbents included Al_2O_3-G Stahl (1090); Al_2O_3-G for TLC, Machery, Nagel Co.; DS-5 Al_2O_3, for TLC, Camag.

The aflatoxins advanced as a single spot in the Al_2O_3 layer and were separated in the silica gel layer. A standard mixture of the aflatoxins, containing 5 ng B_1, 1.5 ng B_2, 5 ng G_1, and 1.5 ng G_2, was spotted and developed by using the miniplate procedure, and a clear separation of the four aflatoxins was obtained.

The method described has been developed for a specific purpose in cotton research. However, the authors believe that a two-layer miniplate method with the same essential features might prove applicable with little or no modification to other commodities in situations in which extreme speed is needed and in which a moderately low limit of detection is satisfactory.

CONCLUSIONS

1. A rapid procedure is described for detecting aflatoxin in field-collected cottonseeds by thin-layer chromatography. It is intended specifically for field situations in developing methods to control aflatoxin contamination of cottonseeds.

2. The procedure incorporates, with modifications, various features of methods described by other workers, with the unique feature of a miniplate coated with two bands of adsorbent, one of aluminum oxide (Al_2O_3) and the other of silica gel.

3. The procedure is supplementary to both slower and more nearly quantitative methods with a large chromatoplate and the faster and somewhat less critical method of detection through the presence of a bright greenish-yellow (BGY) fluorescence in the fiber and seed fuzz.

4. The infection of seeds with other common boll-rot fungi caused no interference in the miniplate detection procedure.

5. An extremely high degree of variability in aflatoxin levels occurred among individual field-collected seeds shown in each case to be infected with Aspergillus flavus.

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